



Cyclin E2 is required for embryogenesis in *Xenopus laevis*

Tetsuya Gotoh¹, Noriko Shigemoto, Takeo Kishimoto^{*}

Laboratory of Cell and Developmental Biology, Graduate School of Bioscience, Tokyo Institute of Technology,
Nagatsuta 4259, Midori-ku, Yokohama 226-8501, Japan

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Abstract

In mammalian cells, E-type cyclins (E1 and E2) are generally believed to be required for entry into S phase. However, in mice, cyclin E is largely dispensable for normal embryogenesis. Moreover, *Drosophila* cyclin E plays a critical role in cell fate determination in neural lineages independently of proliferation. Thus, the functions of cyclin E, particularly during early development, remain elusive. Here, we investigated the requirement for E-type cyclins during *Xenopus* embryogenesis. Although cyclin E1 has been reported as a maternal cyclin, inhibition of its translation in the embryo caused no serious defects. We isolated a *Xenopus* homologue of human cyclin E2, which was zygotically expressed. Sufficient inhibition of its expression led to death at late gastrula, while partial inhibition allowed survival. These observations indicate distinct roles for *Xenopus* cyclins E1 and E2, and an absolute requirement of cyclin E2 for *Xenopus* embryogenesis.

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Introduction

Progression through G1 and S phase in higher eukaryotic cell cycles is widely presumed to be governed by cyclin-dependent kinases (Cdks) associated with D-type and E-type cyclins (Sherr and Roberts, 1999). If so, lack in these cyclins should lead to embryonic lethality. However, recent studies in mice with combinations of disrupted *cyclin* and *Cdk* genes [these disruptions include all three D-type cyclins (D1, D2 and D3), the two E-type cyclins (E1 and E2), cyclin D-dependent Cdk4 and Cdk6, or cyclin E-dependent Cdk2] have revealed that much of fetal development occurs normally in their absence (Berthet et al., 2003, 2006; Geng et al., 2003; Kozar et al., 2004; Malumbres et al., 2004; Ortega et al., 2003; Parisi et al., 2003; Sherr and Roberts, 2004). For example, in the absence of E-type cyclins, mouse embryos can develop until 11.5 days. Although they die thereafter due to failure in endoreplication of placental trophoblasts and loss of giant cells, tetraploid blastocyst rescue

allows most embryos to develop to term (Geng et al., 2003; Parisi et al., 2003). Consistently, the mouse embryonic fibroblast cells derived from these embryos proliferate actively (Geng et al., 2003). Thus, mouse E-type cyclins are largely dispensable for normal embryogenesis. On the other hand, *Drosophila* cyclin E is shown to have a novel function to specify cell fate in neuroblast lineages in the central nervous system independently of cell proliferation (Berger et al., 2005). These somewhat confusing roles of cyclin E during embryogenesis have prompted us to investigate the requirement for cyclin E in amphibian *Xenopus laevis* embryogenesis.

In *Xenopus*, after fertilization, the first twelve cell divisions are rapid and synchronous (Graham and Morgan, 1966). They are characterized by alternating S and M phases, without gap G1 and, except for the first cycle, G2 phases. These cycles are regulated by maternally supplied products, because they occur in the absence of apparent transcription. The midblastula transition (MBT) begins after the twelfth cleavage (Newport and Kirschner, 1982a,b). It is characterized by cell cycle lengthening, loss of cell cycle synchrony, activation of zygotic transcription and appearance of cell motility.

So far, four clones of *Xenopus* cyclin E1, which would be variants from at least two different genes, have been reported by

^{*} Corresponding author. Fax: +81 45 924 5738.

E-mail address: tkishimo@bio.titech.ac.jp (T. Kishimoto).

¹ Present address: Department of Biological Sciences, Derring Hall, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0406, USA.

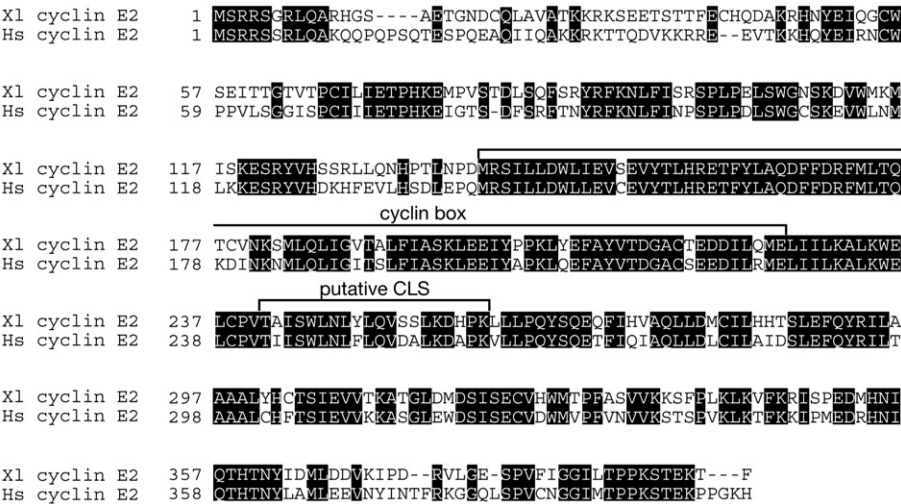


Fig. 1. Sequence analysis of Xcyclin E2. Identical residues between *Xenopus* cyclin E2 (accession No. BC043855) and human cyclin E2 (accession No. AF106690) are boxed in black. Black lines above sequence indicate the cyclin box and a putative centrosome localization sequence (CLS; Matsumoto and Maller, 2004).

two groups (Rempel et al., 1995; Chevalier et al., 1996). Since all of these are the homologue of mammalian cyclin E1 and also are expressed maternally, they are designated as Xcyclin E1 in this paper. During early *Xenopus* embryonic cell cycles, Xcyclin E1 protein is abundant in eggs, remains at elevated levels until the midblastula stage, and then is rapidly degraded at the MBT

(Rempel et al., 1995; Hartley et al., 1996; Howe and Newport, 1996). Such a developmental behavior of Xcyclin E1 suggests the presence of a zygotic cyclin E or a possible homologue of mammalian cyclin E2. However, there has been no report on *Xenopus* cyclin E2, which should be designated as Xcyclin E2. Here, we have cloned Xcyclin E2, and investigated the

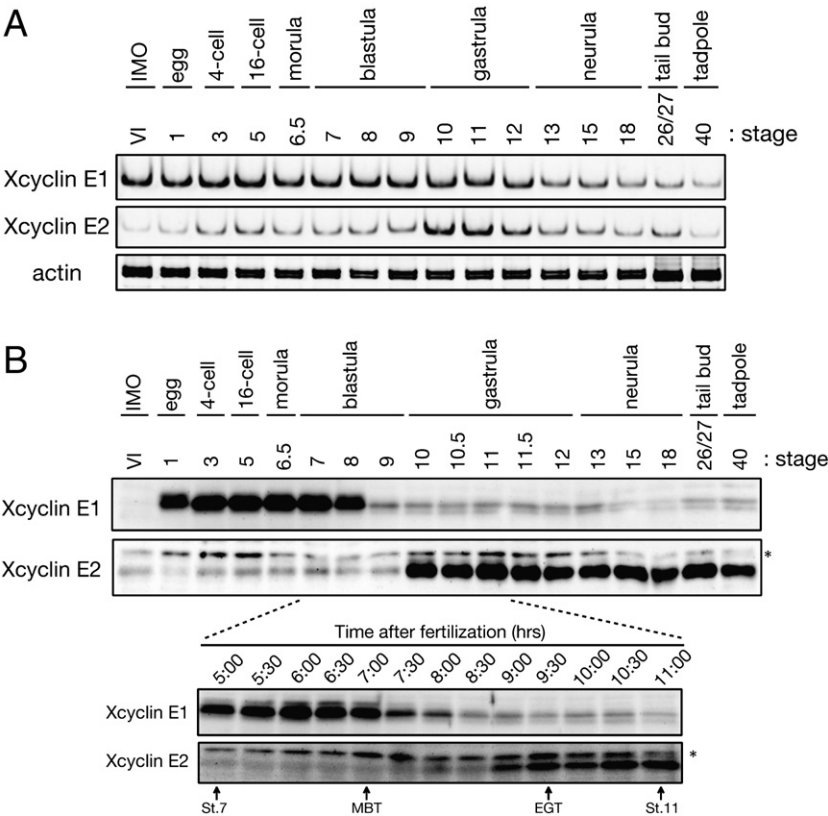


Fig. 2. Xcyclins E1 and E2 are differently expressed during *Xenopus laevis* embryogenesis. (A) RT-PCR was performed on total RNA isolated from immature oocytes (stage VI; IMO) and staged embryos (according to Dumont, 1972; Nieuwkoop and Faber, 1956) using primers specific for either Xcyclin E1 (top), E2 (middle) or control actin (bottom). (B) Western blots with anti-Xcyclin E1 or E2 were performed on extracts from immature oocytes (stage VI) and staged embryos (first and second rows), or from early blastula (stage 7) to mid-gastrula (stage 11) at indicated times after fertilization (third and fourth rows). Each lane was loaded with proteins equivalent to half an embryo. MBT and EGT denote midblastula transition and early gastrula transition, respectively. Asterisk indicates non-specific bands.

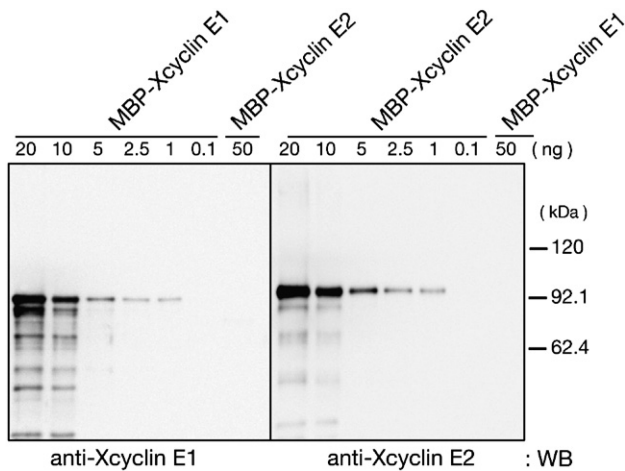


Fig. 3. Specificity of anti-Xcyclin E1 and E2 antibodies. Purified MBP-Xcyclin E1 or MBP-Xcyclin E2 at the indicated amounts was prepared for Western blot analysis (WB) using anti-Xcyclin E1 (left) or anti-Xcyclin E2 (right) antibodies. Note that anti-Xcyclin E1 and Xcyclin E2 antibodies are specific for Xcyclin E1 and Xcyclin E2, respectively, and do not cross-react. Molecular weight markers in kilodalton are indicated on the right side.

requirement for Xcyclin E1 and Xcyclin E2 during *Xenopus* early development. Our results indicate that zygotic expression of Xcyclin E2, but not of Xcyclin E1, is necessary for normal embryogenesis.

Materials and methods

Xenopus oocytes and embryos

Xenopus oocytes and embryos were prepared as described (Gotoh et al., 2001) and staged according to Dumont (1972) and Nieuwkoop and Faber (1956). Morpholino oligonucleotides (MOs; see Figs. 4A and 5A, and Supplementary Fig. 1A) targeted Xcyclin E1 and E2, and standard control MO were purchased from Gene Tools. MOs were injected into fertilized eggs as described (Gotoh et al., 2001).

Cloning of *Xenopus* cyclin E2 cDNA

A BLAST search of the *Xenopus* EST database using DNA sequence of Xcyclin E1 showed an EST sequences (BC043855) having significant homology with human cyclin E2. Full-length cDNA of Xcyclin E2 was generated by PCR against a tailbud cDNA library using PfuUltra High-Fidelity DNA polymerase (Stratagene).

Construction of recombinant plasmids

To obtain pGEX-Xcyclin E2, pMAL-Xcyclin E2, pGEX-Xcyclin E1 or pMAL-Xcyclin E1, fragments that were created by PCR using respective type of

Xcyclin E cDNA were cloned into pGEX-4T-1 (Amersham) or pMAL (NewEngland Biolabs). Bacterially produced GST or MBP recombinant was expressed and purified according to Gotoh et al. (2001).

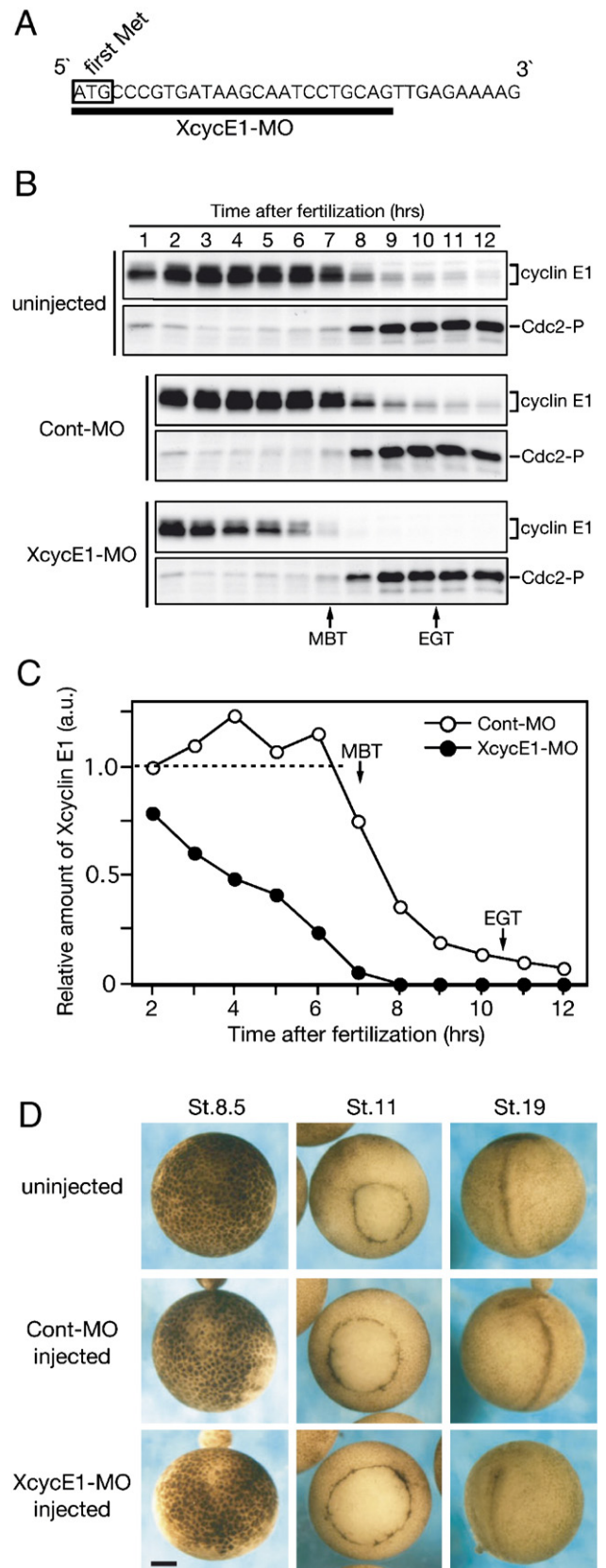


Fig. 4. Xcyclin E1 is dispensable for development after MBT. (A) A morpholino oligo for Xcyclin E1 (XcycE1-MO) was targeted to the underlined sequence. The box indicates the start codon. (B) One-cell stage embryos were uninjected or injected with 80 ng of either control MO (Cont-MO) or XcycE1-MO, cultured, collected at indicated times, and analyzed by Western blot with anti-Xcyclin E1 or anti-phospho-Cdc2-Tyr15 (Cdc2-P) antibodies. (C) The Xcyclin E1 signals shown in panel B were quantified by phosphoimager. (D) Embryos as in panel B were photographed when uninjected control embryos reached the indicated stages. Note that neural tube was not still closed in XcycE1-MO-injected embryos when uninjected embryos reached stage 19. Scale bar, 250 μ m.

Specific antibodies and immunoblotting

Rabbits were immunized with bacterially produced GST-Xcyclin E1 or E2. Polyclonal antibodies were purified using membranes onto which GST-Xcyclin E1 or E2 had been transferred. Western blot analysis was performed as described (Gotoh et al., 2001). The primary antibodies used were anti-Xcyclin E1, anti-Xcyclin E2, anti-phospho-human Cdc2 (Tyr-15) (New England Biolabs), and anti-MAPK (Upstate). The secondary antibodies were HRP-conjugated secondary antibody (Amersham) and alkaline phosphatase-conjugated secondary antibodies (Dako). Signals were visualized by the ECL-plus system (Amersham) or BCIP/NBL phosphatase substrate system (KPL).

RT-PCR analysis

RNA was extracted from oocytes and embryos using Trizol reagent (Invitrogen) and treated with RNase-free DNase I (Takara). cDNA was synthesized from the extracted RNA using oligo-dT12-16 and Superscript II (Invitrogen). Aliquots of the reaction products were subjected to PCR (95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min) for 25 cycles; the 5' and 3' PCR primers were respectively, 5'-GATCTTGGTTGGTGCTTAAGTCCGATGAC-3' and 5'-GTAGGTATAGGTGAGGTCTATTCTGCTCC-3' for Xcyclin E1, 5'-GCTTTGAAGTGGGAATTGTGTCTCTGTAAC-3' and 5'-CCAATGAACA-CAGGAGACTCACCCAAAAC-3' for Xcyclin E2, and 5'-GAAGCCCCAAG-CAGAGAGGTATCC-3' and 5'-ATCTGCTGGAAAGTGGAGAGTGAGG-3' for *Xenopus* β -actin type 8. Reaction products were fractionated on acrylamide gels and stained with SyberGold Green I (Molecular Probes).

Results

Xenopus cyclin E2 is a zygotic type of cyclin E

To clone a potential *Xenopus* homologue of mammalian cyclin E2, we performed a BLAST search of *Xenopus* EST database using DNA sequence of Xcyclin E1. We found an EST sequence (BC043855) which encodes another full-length *Xenopus* cyclin E protein. This cyclin E had 397 amino acids (Fig. 1), and the predicted molecular mass was 45.7 kDa. Since it showed more significant homology with human cyclin E2 than human cyclin E1 or *Xenopus* cyclin E1 (63% vs. 45% or 42% identity; see Supplementary Table 1), we called it *Xenopus* cyclin E2 (Xcyclin E2). Xcyclin E2 cDNA was then isolated by PCR against cDNA library derived from tailbud and its nucleotide sequence was confirmed.

To examine the expression pattern of Xcyclin E mRNA, we performed semiquantitative RT-PCR analysis. Xcyclin E1 mRNA was confirmed to be present at a relatively constant level until late gastrula as reported previously (Rempel et al., 1995; Fig. 2A). By contrast, levels of Xcyclin E2 mRNA were found to be low until late blastula stage, followed by increase during gastrula stage (Fig. 2A). This observation indicates that expression of Xcyclin E2 mRNA is regulated by zygotic transcription after the MBT, raising a possibility that Xcyclin E2 protein is a zygotic type of cyclin E.

We then generated polyclonal antibodies against Xcyclin E1 or E2. Anti-Xcyclin E1 and E2 antibodies specifically recognized recombinant MBP-Xcyclin E1 and E2, respectively, and did not cross-react (Fig. 3). Western blot analysis using anti-Xcyclin E1 antibodies confirmed previous reports (Rempel et al., 1995; Hartley et al., 1996; Howe and Newport, 1996): levels of Xcyclin E1 protein remained high until onset of the MBT,

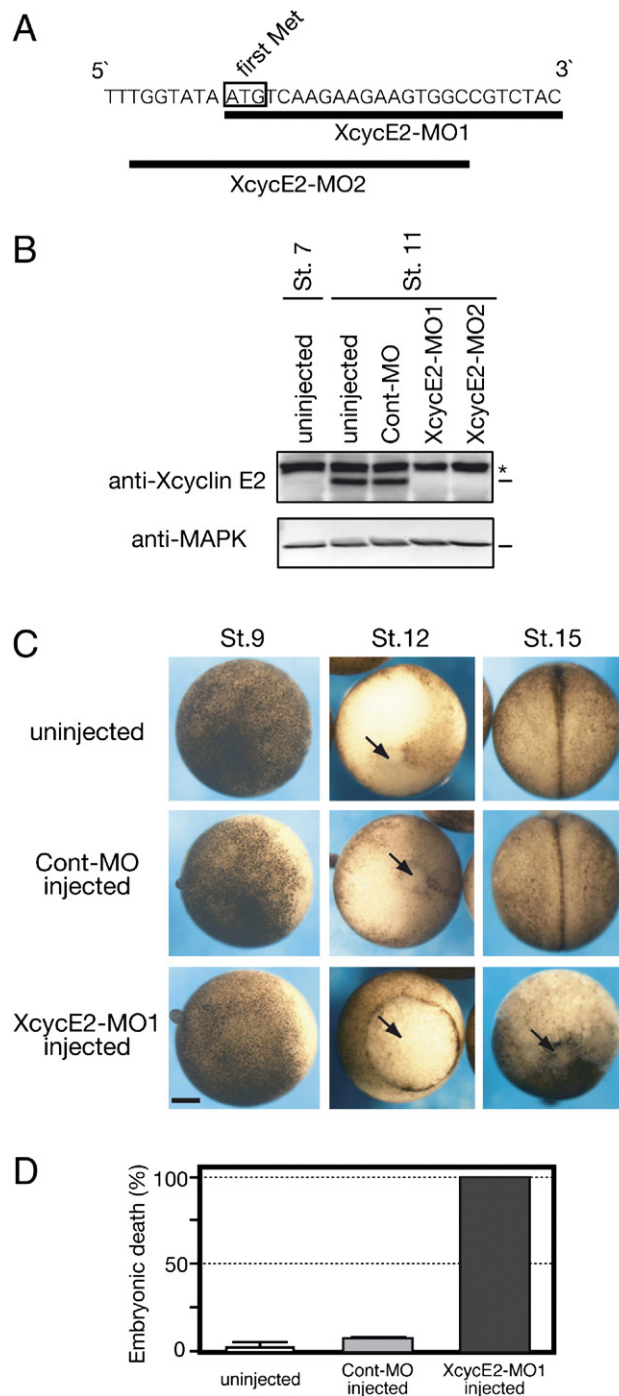


Fig. 5. Sufficient loss of Xcyclin E2 is lethal during late gastrulation. (A) Two types of morpholino oligos for Xcyclin E2 (XcycE2-MO1 and -MO2) were targeted to underlined sequences, respectively. The box indicates the start codon. (B) One-cell stage embryos were uninjected or injected with 80 ng of either Cont-MO, XcycE2-MO1 or XcycE2-MO2, cultured, collected when uninjected embryos reached at stages 7 and 11, and analyzed by Western blot with anti-Xcyclin E2 (top) or control anti-MAPK antibodies (bottom). Asterisk indicates non-specific bands. (C) Embryos as in panel B were photographed when uninjected embryos reached the indicated stages. Arrows indicate blastopore. Scale bar, 250 μ m. (D) Embryos as shown in panel B were inspected for embryonic death when uninjected embryos reached stage 15. The error bar represents the standard deviation (SD) from triplicate samples.

then rapidly decreased, and thereafter, remained low (Fig. 2B). In contrast, levels of Xcyclin E2 protein were found to be very low until blastula stage, greatly increased around early gastrula, and then remained constant up to the tadpole stage (Fig. 2B). These observations indicate that Xcyclin E2 protein is largely expressed as a zygotic type of cyclin E (see also Fig. 5B).

Translation of cyclin E1 mRNA after fertilization is not required for Xenopus embryogenesis

To investigate requirement for Xcyclin E1 and E2 in early development, an Xcyclin E1-targeted antisense morpholino oligo (XcycE1-MO; see Fig. 4A) was first injected into fertilized eggs. XcycE1-MO reduced Xcyclin E1 protein levels soon after the injection depending on its dose (data not shown), resulting in complete disappearance of Xcyclin E1 protein by 8 h after fertilization (Figs. 4B, C). XcycE1-MO had no effect on inhibitory Tyr15 phosphorylation of Cdc2 at the MBT, an indicator of the cell cycle elongation (Fig. 4B). Microscopic inspection of the developing embryos indicated that the Xcyclin E1 knockdown did not affect developmental events such as gastrulation and neurulation, except for slight delay in development (Fig. 4D). The same dose of commercial standard control morpholino oligo (Cont-MO) had no effect (Figs. 4B–D). Thus the Xcyclin E1 knockdown did not cause any serious defects, as reported previously (Slevin et al., 2005).

Cyclin E2 is required for Xenopus embryogenesis

We next examined whether the knockdown of Xcyclin E2 has any effect on early development. For this purpose, each of two types of Xcyclin E2-targeted antisense morpholino oligonucleotides (XcycE2-MO1 and -MO2; see Fig. 5A) was injected into eggs immediately after fertilization. XcycE2-MO1 and -MO2 almost completely inhibited the expression of Xcyclin E2 (Fig. 5B). The effect of XcycE2-MOs was generally dose-dependent and the same dose of Cont-MO had no effect (data not shown). XcycE2-MO1-injected embryos underwent cell division normally until late blastula stage (Fig. 5C). Then, however, they tended to delay in the progression of gastrulation (Fig. 5C), although we could not recognize whether cell divisions occurred normally or not because the size of each blastomere became smaller during embryogenesis. Thereafter, the embryos died at the end of gastrulation with a dramatic disruption of intercellular contacts, which is characteristic of apoptosis (Hensey and Gautier, 1997) (Fig. 5C). When intact embryos and Cont-MO-injected embryos reached at mid-neurula stage, all of the XcycE2-MO1-injected embryos died (Fig. 5D). The same deleterious effect was observed also in the XcycE2-MO2-injected embryos (data not shown). Thus, almost complete loss of Xcyclin E2 was ultimately lethal to the embryo, suggesting that Xcyclin E2 is required for normal development from gastrula to neurula.

Although rescue experiments would further support our conclusions, it was not practical to perform these due to the following reasons. First, overlap of both XcycE2-MO1 and

-MO2 with the coding region of Xcyclin E2 protein (Fig. 5A) excludes injection of a possible Xcyclin E2 mRNA construct that escapes from inhibition by the antisense morpholino oligos. Second, injection of recombinant Xcyclin E2 protein at the one-cell stage might not be appropriate, because increase in endogenous Xcyclin E2 protein levels first occurs around early gastrula stage (Fig. 2B).

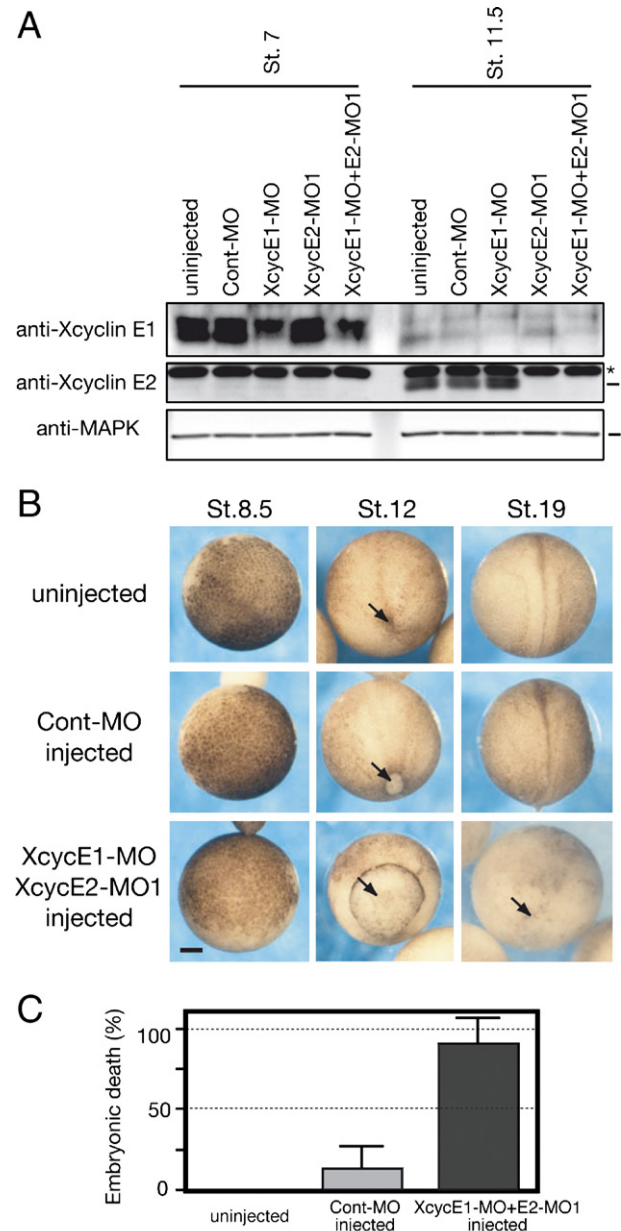


Fig. 6. Sufficient loss of both Xcyclin E1 and E2 is embryonic lethal at late gastrulation. (A) One-cell stage embryos were uninjected or injected with 160 ng of Cont-MO, 80 ng of XcycE1-MO or XcycE2-MO1, or each 80 ng of both XcycE1-MO and XcycE2-MO1, cultured, collected when uninjected embryos reached stages 7 (early blastula) and 11.5 (late gastrula), and analyzed by Western blot with anti-Xcyclin E1 (top), anti-Xcyclin E2 (middle) or control anti-MAPK antibodies (bottom). Asterisk indicates non-specific bands. (B) Embryos as in panel A were photographed when uninjected embryos reached the indicated stages. Arrows indicate blastopore. Scale bar, 250 μ m. (C) Embryos as in panel A were inspected for embryonic death when uninjected embryos reached stage 15. The error bar represents the SD from triplicate samples.

In contrast to the case of XcycE2-MO1 and -MO2, incomplete inhibition of Xcyclin E2 expression by XcycE2-MO3 did not affect the timing of developmental events such as gastrulation and neurulation (data not shown). However, preliminary observation recognized visible morphological defects such as formation of a small head with small or no eyes, when development was followed up to the tadpole stage (Supplementary Fig. 1). At present, it is unclear whether these morphological defects implicate a non-cell cycle role for cyclin E which is reported in segment-specific neural lineages of *Drosophila* (Berger et al., 2005). In any case, it is likely that the transition from gastrula to neurula is allowed if Xcyclin E2 is expressed partially.

Finally, we performed the double knockdown of Xcyclin E1 and E2 by injecting both XcycE1-MO and XcycE2-MO1 into fertilized eggs. They inhibited the translation of Xcyclin E1 and E2 mRNA (Fig. 6A). Phenotype of the double-knockdown embryos was the same as that of the single Xcyclin E2 knockdown embryos (Figs. 6B, C). Thus, almost complete loss of Xcyclin E2 led to the embryonic death at the end of gastrulation, regardless of the presence (see Fig. 2B) or the absence of low levels of Xcyclin E1 after MBT. That is, low levels of Xcyclin E1 after MBT were dispensable even in the absence of cyclin E2.

Discussion

The present study shows that in normal embryogenesis of *X. laevis* up to the tadpole stage, Xcyclin E1 protein remains at low levels after its large disappearance at the blastula stage, while Xcyclin E2 protein is largely expressed from the early gastrula stage; and that after the MBT, Xcyclin E1 is not essential for embryogenesis up to the tadpole stage, while Xcyclin E2 is indispensable for survival of embryos to the late gastrula. These observations indicate that Xcyclin E2 is the first zygotic type of cyclin E to be identified in *X. laevis*, and that E1- and E2-type cyclins have distinct roles during embryogenesis.

A compelling question is what is a role for Xcyclin E2 during the gastrula stage. In relation to this, there are interesting reports about the effects of aphidicolin, a DNA replication inhibitor, on *Xenopus* embryogenesis (Harris and Hartenstein, 1991; Rollins and Andrews, 1991; Carter and Sible, 2003): when the inhibitor is added either to mid- or late blastula, the embryos continue to develop with slight delay and then die around early or late gastrula, respectively, while when it is added during neurulation, the embryos continue to differentiate almost normally up to the tadpole stage. These previous observations imply that for *Xenopus* embryogenesis, S phase is essential during blastula and gastrula stages, and its prevention results in embryonic lethality. Considering that full, but not partial, inhibition of Xcyclin E2 expression caused the late gastrula death in the present study, Xcyclin E2 is most likely to be required for entry into S phase at least during gastrula stage, but partial levels of its expression might meet this requirement. A possible cause for the late gastrula death would be the activation of the DNA replication checkpoint

(Kappas et al., 2000; Gotoh, unpublished), although it remains unclear whether the checkpoint activation actually induces cell death in *Xenopus* gastrula.

When Xcyclin E2 expression was sufficiently suppressed, the late gastrula death occurred even though Xcyclin E1 protein was expressed at low levels. Consistently, sufficient double knockdown of Xcyclin E1 and E2 and a single knockdown of Xcyclin E2 showed the similar lethal phenotype (Figs. 5 and 6). Nonetheless, the death was evaded when Xcyclin E2 was partially expressed (Supplementary Fig. 1). These observations suggest that a role of Xcyclin E2 for the late gastrula survival cannot be compensated by Xcyclin E1.

In conclusion, Xcyclin E2 is a novel zygotic type of cyclin E and is essential for the late gastrula survival during embryogenesis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ydbio.2007.08.005](https://doi.org/10.1016/j.ydbio.2007.08.005).

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